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(54) Title: INTRODUCING NUCLEIC ACID INTO ANIMAL CELLS

(57) Abstract

    A reagent for transfecting a nucleic acid into an animal cell containing a neutral phospholipid such as dioleoylphosphatidylethanolamine, a cationic lipid such as stearylamine or dimethyldioctadecylammonium bromide, a tertiary carbyl amine, or a benzethonium salt such as methylbenzethonium chloride. A method for using this reagent in the transfection process is also described.

INTRODUCING NUCLEIC ACID INTO ANIMAL CELLS

GOVERNMENT RIGHTS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention concerns a reagent and a method for introducing nucleic acid into animal cells.

Background Information

There are currently four major reagents or methods used to introduce DNA into animal cells. These are (1) $CaPO_4$-DNA precipitates, (2) DEAE dextran-DNA complexes, (3) electroporation and (4) "LIPOFECTIN"[TM] reagent, a transfection reagent marketed by BRL (Life Technologies, Inc., Gaithersburg, Maryland).

Recently, a liposome-mediated transfection protocol ("LIPOFECTION"[TM]) has been reported for the introduction of DNA into animal cells (Philip L. Felgner, Thomas R. Gadek, Marilyn Holm, Richard Roman, Hardy W. Chan, Michael Wenz, Jeffrey P. Northrop, Gordon M. Ringold and Mark Danielsen, "Lipofection: A Highly Efficient, Lipid-Mediated DNA-Transfection Procedure", Proc. Natl. Acad. Sci. USA, 84, 7413-7417 (1987)). This protocol uses the synthetic cationic lipid DOTMA (N-[1-(2,3-

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dioleyloxy)propyl]-N,N,N-trimethyl-ammonium chloride).
Liposomes composed of DOTMA and a neutral lipid PtdEtn
(dioleoylphosphatidylethanolamine) form stable complexes
with DNA, and deliver DNA into several eukaryotic cells with
higher efficiency and reproducibility than other methods.

A transient expression system that requires
transfected DNA to be present in the cytoplasm has recently
been described by T.R. Fuerst, E.G. Niles, W. Studier and B.
Moss, Proc. Natl. Acad. Sci. USA, 83, 8122-8126 (1986).
This system is based on use of a recombinant vaccinia virus
that synthesizes bacteriophage T7 RNA polymerase. The
plasmid DNA containing the gene of interest under control of
the T7 promoter is transfected as a $CaPO_4$ precipitate into
the cytoplasm of the vaccinia infected cells where it is
transcribed efficiently by the T7 RNA polymerase. The mRNA
derived from the transfected gene can be as much as 10% of
the total cytoplasmic RNA. This system has facilitated
studies of viral glycoprotein translocation (A.S. Shaw,
P.J.M. Rottier and J.K. Rose, Proc. Natl. Acad. Sci. USA,
85, 7592-7596, (1988)) and virus assembly (M. Whitt, L.
Chong and J.K. Rose, J. Virol., 63, 3569-3578 (1989)), and
has allowed for the definition of interacting domains of the
lymphocyte glycoprotein CD4 and an intracellular tyrosine
protein kinase (A. Shaw, K. Amrein, C. Hammond, D.F. Stern,
B.M. Sefton and J.K. Rose, Cell, 59, 627-636 (1989)). A
major difficulty with this system was the lack of

reproducibility of the transfection step using $CaPO_4$

precipitates of DNA (Fuerst et al, <u>Proc. Natl. Acad. Sci.</u>

<u>USA</u>, <u>83</u>, 8122-8126, (1986) and F.L. Graham and A.J. Van Der

Eb, <u>Virology</u>, <u>52</u>, 456-467, (1973)). The percentage of cells

expressing protein showed a high degree of day-to-day

variability, some plasmid DNA preparations were inactive for

unknown reasons, and it was not possible to use impure

plasmid DNA preparations containing large amounts of RNA

(DNA from minipreps).

The variability in the transfection was overcome

by using lipofection instead of the $CaPO_4$ procedure (M.

Whitt et al, <u>J. Virol.</u>, <u>63</u>, 3569-3578, (1989)) and a three-

fold increase over the best expression levels obtained with

$CaPO_4$ was found.

A major drawback to the DOTMA transfection

procedure is that the compound itself is not commercially

available, and the preformed liposomes containing DOTMA

("LIPOFECTIN"™ reagent, Life Technologies, Inc.,

Gaithersburg, Maryland) are too expensive for large scale

use in transient assays. The cost of lipofection is

prohibitive ($145/ml or about $10 per transfection) to

laboratories, especially to laboratories which perform

thousands of transfections per year.

P.L. Felgner and G.M. Ringold, "Cationic Liposome-

Medicated Transfection", <u>Nature</u>, <u>337</u>, 387-388, (1989) at

page 387 report that liposomes comprised of stearylamine or

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dioctaldecyl-dimethylammonium bromide were inactive in
transfection assays.

J.-P. Behr, B. Demeneix, J-P. Loeffler and J.P.
Mutul, Proc. Natl. Acad. Sci., USA, 86, 6982-6986, (1989)
described a transfection procedure using compacted
lipopolyamine-coated plasmids.

It would be advantageous to have a reagent and
method for introducing nucleic acids into animal cells using
readily available and relatively inexpensive compounds.

SUMMARY OF THE INVENTION

It is an object of the present invention to
introduce nucleic acids into animal cells using relatively
available and relatively inexpensive reagents. This object,
as well as other objects, aims and advantages are achieved
by the present invention.

The present invention concerns a reagent for
introducing nucleic acids into an animal cell comprising

a.   a neutral lipid, for example, dioleyl
phosphatiylethanolamine, and

b.   a cationic lipid selected from the group
consisting of

(1)   an ammonium salt of the formula

$$\begin{bmatrix} R_1 \!-\!\!-\!\!- \underset{\underset{R_4}{|}}{\overset{\overset{R_2}{|}}{N}} \!-\!\!-\!\!- R_3 \end{bmatrix} \quad [A]$$

, wherein R_1 is a straight hydrocarbon chain of C_{14} to C_{18} that is saturated or unsaturated,

R_2, R_3 and R_4 are, independently of each other, hydrogen, a straight hydrocarbon chain of C_1 to C_{18} that is saturated or unsaturated or an aryl, e.g., benzyl or phenyl, and A is an anion,

(2)   an amine of the formula

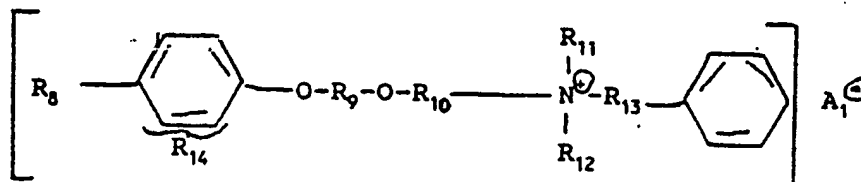$$R_5\text{---}N\text{---}R_7$$

with R_6 above N, wherein R_5 is a straight chain of C_{14} to C_{18} that is saturated or unsaturated and wherein R_6 and R_7, independently of each other, are hydrogen or C_1 to C_5 alkyl and

(3)   a benzethonium salt of the formula

$$\left[R_8\text{---}\langle\text{---}\rangle\text{---}O\text{-}R_9\text{-}O\text{-}R_{10}\text{---}N^{\oplus}\text{-}R_{13}\text{---}\langle\text{---}\rangle \right] A_1^{\ominus}$$

with R_{11}, R_{12} on the nitrogen and R_{14} on the first ring.

, wherein R_8 is a straight chain or branched C_1-C_{10}-alkyl,

R_9 is a C_1-C_{10}-alkyl,

R_{10} is a C_1-C_{10}-alkyl,

R_{11} is a C_1-C_{10}-alkyl,

R_{12} is a C_1-C_{10}-alkyl,

R_{13} is a C_1-C_{10}-alkyl,

R_{14} is a C_1-C_{10}-alkyl and

$A_1^{\ominus}$ is an anion.

The present invention also concerns a method for introducing a nucleic acid into an animal cell comprising

     a.   mixing nucleic acid with a reagent as described above to form a liposome or lipid micelles and

     b.   contacting the resultant liposome or lipid micelles with an animal cell.

The present invention further relates to a complex between a nucleic acid and the reagent as defined above. Still further, the present invention is directed to such complex further comprising a liposome or lipid micelles.

BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention there is shown in the drawings forms which are presently preferred. It is to be understood, however, that the present invention is not limited to the precise arrangements and instrumentalities depicted in the drawings.

Fig. 1 depicts flow cytometry profiles showing a high frequency of VSV glycoprotein expression on the surface of HeLa cells. Fig. 1A is a profile for a control (mock transfection). Fig. 1B is a profile for pARG transfected (pARG is a plasmid that encodes a viral glycoprotein).

Fig. 2 is a series of bar graphs depicting the effect of DDAB concentration on expression frequency for pARG transfected for BHK cells and HeLa cells.

Fig. 3 is a series of bar graphs depicting the effect of DNA concentration on expression frequency for pARG transfected in BHK cells.

Fig. 4 is a graph showing the effect of DNA concentration on the rate of VSV G protein synthesis.

Fig. 5 depicts flow cytometry profiles for a control (mock transfected) (Fig. 5A), DDAB (Fig. 5B), "LIPOFECTIN"™ reagent that contains DOTMA (Fig. 5C) and CaPO₄ (Fig. 5D).

DETAILED DESCRIPTION OF THE INVENTION

The invention concerns a reagent and a method for introducing nucleic acid into animal cells with very high efficiency. The reagent is composed of a cationic lipid (detergent), for example, dimethyldioctadecylammonium bromide, and a neutral lipid, for example, dioleoyl phosphatidylethanolamine. Liposomes are formed from these two compounds. Nucleic acid, e.g., DNA or RNA, is mixed with the preformed liposomes in medium and added to cells, e.g., on a tissue culture dish. This results in nucleic acid, e.g., DNA, delivery into the cells with high efficiency.

Non-limiting examples of other neutral lipids for use in the present invention include the following: phospholipid-related materials such as lecithin, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphinogomyelin, cephalin, cardiolipin, phosphatidic acid, cerebrosides, acetylphosphate, dioleoylphosphatidylcholine (DOPE),

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dipalmitoylphosphatidylcholine, dioleoylphosphatidyl-
glycerol (DOPC), dipalmitoylphosphatidylglycerol,
dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-
phosphatidylcholine (POPC), palmitoyloleoylphosphatidyl-
ethanolamine (POPE), dioleoylphosphatidylethanolamine 4-(N-
maleimidomethyl) cyclohexane-1-carboxylate (DOPE-MAL),
diheptadecanoyl phosphatidylethanolamine, dilauroylphos-
phatiylethanolamine, dimyristoylphosphatidylethanolamine,
distearoyl phosphatidylethanolamine, beta-linoleoyl-gamma-
palmitoyl phosphatidylethanolamine and beta-oleoyl-gamma-
palmitoyl phosphatidylethanolamine.

The cationic lipid for use in the present
invention is

(1) an ammonium salt of the formula

$$\left[R_1 - \underset{\underset{R_4}{|}}{\overset{\overset{R_2}{|}}{N}} - R_3 \right] \quad [A]$$

, wherein R_1 is a straight hydrocarbon chain of C_{14} to C_{18}
that is saturated or unsaturated,

R_2, R_3 and R_4 are, independently of each other,
hydrogen, a straight hydrocarbon chain of C_1-C_{18} that is
saturated or unsaturated, preferably C_1-C_5-alkyl or C_{18}-alkyl
or an aryl, e.g., a benzyl or phenyl and A is an anion, for
example, a halogen, for example, Br, Cl, I or F, preferably
Br, or a sulfate, nitrite or nitrate, wherein preferred
embodiments of such salt are cetyldimethyethylammonium

bromide of the formula $CH_3(CH_2)_{15}N(CH_3)_2(C_2H_5)Br$ and

dimethyldioctadecylammonium bromide (DDAB) of the formula

$((CH_3)(CH_2)_{17})_2N(CH_3)_2Br$,

(2) an amine of the formula

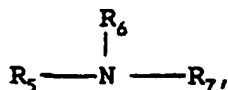$$R_5 \underset{\overset{\displaystyle |}{\underset{\displaystyle}{}}}{\overset{\displaystyle R_6}{\text{—N—}}} R_7,$$

wherein R_5 is a straight chain of C_{14} to C_{18} that is saturated

or unsaturated and wherein R_6 and R_7, independently of each

other, are hydrogen or C_1 to C_5 alkyl, preferably the amine

is stearylamine of the formula $CH_3(CH_2)_{17}NH_2$, or

(3) a benzethonium salt of the formula

, wherein R_8 is a straight chain or branched C_1-C_{10}-alkyl,

R_9 is a C_1-C_{10}-alkyl,

R_{10} is a C_1-C_{10}-alkyl,

R_{11} is a C_1-C_{10}-alkyl,

R_{12} is a C_1-C_{10}-alkyl,

R_{13} is a C_1-C_{10}-alkyl,

R_{14} is a C_1-C_{10}-alkyl and $A_1^{\ominus}$ is an anion, for example, a

halogen, for example, Br, Cl, I or F, preferably Cl, or a

sulfate, nitrite or nitrate, preferably the benzethonium

salt is methylbenzethonium chloride of the formula

$$\left[(CH_3)_3 \, C \, CH_2 \, C(CH_3)_2 - \langle \!\!\!\! \rangle - OCH_2CH_2OCH_2CH_2 - \overset{\oplus}{\underset{CH_3}{\overset{CH_3}{N}}} - CH_2C_6H_5 \right] Cl^-$$

(N,N-dimethyl-N-[2-[2-methyl-4-(1,1,3,3-tetramethylbutyl)-phenoxy]ethoxy]ethyl]benzenemethanaminium chloride).

The present invention also encompasses substituted forms of the ammonium salts, amines and benzethonium salts as described above, wherein the use of such substituted forms in the reagent functions to allow the introduction of nucleic acid into an animal cell.

The ratio of cationic lipid to neutral lipid can be widely varied, depending on the particular cationic lipid employed. Thus for cetyldimethylethylammonium bromide and methylbenzethonium chloride the ratio can be 3/30 to 12/30; for dimethyldioctadecylammonium bromide the ratio can be 6/30 to 12/30; and for stearylamine, the ratio can be 0.3/30 to 30/30.

The ratio of nucleic acid, e.g., DNA, to cationic lipid should not be so high as to saturate the positive charges on the liposomes, which could result in that the liposomes would not bind the cell surface.

Animal cells for use in the present invention include cells from humans and non-human warm blooded animals. Erythrocytes, for example, can be employed. Also baby hamster kidney (BHK) cells, AtI 20 cells, RK-13 cells and Madin-Darby canine kidney (MDCK) cells can be used in the present invention.

Although cytoplasmic gene expression is preferred, gene expression in the cell nucleus can be conducted according to the invention.

Nucleic acid as used in this application includes DNA, RNA and oligonucleotides of DNA and RNA.

The nucleic acid, e.g., DNA or RNA, coding for specific genes of interest may be retrieved, without undue experimentation, from the GenBank of EMBL DNA libraries. Such sequencs may include coding sequences, for example, the coding sequences for structural proteins, hormones, receptors and the like, and the DNA sequencces for other DNAs of interest, for example, transcriptional and translational regulatory elements (promoters, enhancers, terminators, signal sequnces and the like), vectors (integrating or autonomous) and the like. Non-limiting examples of DNA sequences which may be introduced into cells with the reagent of the invention include those sequences coding for fibroblast growth factor (WO 87/01728); ciliary neurotrophic factor (Lin et al, Science, 246:1023-1025 (1989); human interferon-α receptor (Uzé, G. et al, Cell, 60:225-235 (1990); the interleukins and their receptors (reviewed in Mizal, S.B., FASEB J., 3:2379-2388 (1989); hybrid interferons (see European Patent Application No. 051,873); the RNA genome of human rhinovirus (see Callahan, P.L., Proc. Natl. Acad. Sci. (USA), 82:732-736 (1985)); antibodies including chimeric antibodies (see Cabilly et al,

U.S. Patent 4,816,567); reverse transcriptase (see Moelling,
K., et al, J. Virol., 32:370-378 (1979)); human CD4 and
soluble forms thereof (Maddon et al, Cell, 47:333-348
(1986); PCT Application Publication Nos. WO 88/01304 (1988)
and WO 89/01940 (1989)). See also Seed, B. et al, European
Patent Application Publication No. 330,191, who disclose a
rapid immunoselection cloning method which is useful for the
cloning of a large number of important proteins. The
disclosures of the references cited above are fully
incorporated by reference herein.

The present invention can be employed for the
introduction of DNA, for example, into animal cells for
research purposes. The most common reason for doing this is
to obtain transient or permanent expression of DNA in
established animal cell lines. This is done on a daily
basis in thousands of laboratories worldwide.

The present invention can also be employed in gene
therapy, e.g., to treat genetic diseases in humans and non-
human warm blooded animals. For example, a "missing gene"
can be introduced in the bone marrow of a patient by the
present invention.

The invention can further be used to treat
infectious diseases, e.g., AIDS, by blocking infection.

Transformation/Transfection is defined as follows:
the introduction of DNA or RNA into cells in such a way as
to allow gene expression.

Liposomes consist of spheres of lipid bilayers (two-molecules thick) that enclose an aqueous medium.

Liposomes can generally be formed by sonicating a lipid in a aqueous medium, by resuspension of dried lipid layers in a buffer or by dialysis of lipids dissolved in an organic solvent against a buffer of choice.

Phospholipids form closed, fluid-filled spheres when they are mixed with water, in part because the molecules are amphipathic: they have a hydrophobic (water-insoluble) tail and a hydrophilic (water-soluble), or "polar," head. Two fatty acid chains containing up to about 24 carbon atoms generally make up the hydrophobic tail of most naturally occurring phospholipid molecules. Phosphoric acid bound to any of several water-soluble molecules composes the hydrophilic head. When a high enough concentration of phospholipids is mixed with water, the hydrophobic tails spontaneously herd together to exclude water, whereas the hydrophilic heads bind to water.

The result is a bilayer in which the fatty acid tails point into the membrane's interior and the polar head groups point outward. The polar groups at one surface of the membrane point toward the liposome's interior and those at the other surface point toward the external environment. As a liposome forms, any water-soluble molecules that have been added to the water are incorporated into the aqueous spaces in the interior of the spheres, whereas any lipid-

soluble molecules added to the solvent during vesicle

formation are incorporated into the lipid bilayer.

Liposomes typically range in diameter from 250

angstrom units to several micrometers (the diameter of a red

blood cell is roughly 10 micrometers) and are usually

suspended in a solution. They have two standard forms:

"onion-skinned" multilamellar vesicles (MLV's), made up of

several lipid bilayers separated by fluid, and unilamellar

vesicles, consisting of a single bilayer surrounding an

entirely fluid core. The unilamellar vesicles are typically

characterized as being small (SUV's) or large (LUV's). The

unilamellar vesicles are preferred in the present invention.

Under appropriate circumstances liposomes can

adsorb to almost any cell type. Once they have adsorbed the

spheres, liposomes may be endocytosed, or swallowed up, by

some cells. Adsorbed liposomes can also exchange lipids

with cell membranes and may at times be able to fuse with

cells. When fusion takes place, the liposomal membrane is

integrated into the cell membrane and the aqueous contents

of the liposome merge with the fluid in the cell.

Endocytosis of liposomes occurs in a limited class

of cells; those that are phagocytic, or able to ingest

foreign particles. When phagocytic cells take up liposomes,

the cells move the spheres into subcellular organelles known

as lysosomes, where the liposomal membranes are thought to

be degraded. From the lysosome, the liposomal lipid

components probably migrate outward to become part of the cell's membranes and other liposomal components that resist lysosomal degradation (such as certain medications) may enter the cytoplasm.

Lipid exchange involves the transfer of individual lipid molecules from the liposome into the plasma membrane (and vice versa); the aqueous contents of the liposome do not enter the cell. For lipid exchange to take place, the liposomal lipid must have a particular chemistry in relation to the target cell. Once a liposomal lipid joins the cell membrane it can either remain in the membrane for a long time or be redistributed to a variety of intracellular membranes.

In very dilute solutions, lipid micelles may form instead of liposomes.

With respect to screening procedures described herein, alternative components active in transfection could be screened by substituting them for PtdEtn in the preparation of liposomes containing cationic lipids. These liposomes would then be used to transfect cells with plasmid DNA encoding a protein that is easily assayed (for example the VSV glycoprotein). The frequency of gene expression in cells would be determined by flow cytometry as depicted in Fig. 1 and Fig. 5.

The invention will now be described with reference to the following non-limiting examples.

Examples

Materials

Lipids

L-α-dioleoylphosphatidylethanolamine (PtdEtn),
stearylamine, cetyldimethlyethyl-ammonium bromide,
dimethyldioctadecylammonium bromide (DDAB), and
methylbenzethonium chloride (N,N-dimethyl-N-[2-(2-[methyl-4-
(1,1,3,3-tetramethylbutyl)-phenoxy]ethoxy)ethyl]-
benzenemethanaminium chloride), were purchased from Sigma
Chemical Co., St. Louis, Mo. "LIPOFECTIN"™ reagent was
purchased from Life Technologies, Inc., Gaithersberg, Md.

Cells and Media

HeLa cells were grown in Dubecco-Vogt's modified
minimal essential medium (DMEM) containing 10% horse serum
or 5% fetal calf serum. Baby Hamster Kidney (BHK) cells,
AtT-20 cells, RK-13 cells, and Madin-Darby canine kidney
cells (MDCK) cells were grown in DME containing 5% fetal
calf serum.

Example 1: Liposome Preparation

Dioleoylphosphatidylethanolamine (PtdEtn) was
supplied as a 10mg/ml solution in $CHCl_3$. The other lipids
were dissolved in $CHCl_3$ at 100mg/ml. Liposomes were
typically prepared as follows. One mg of PtdEtn (0.1 ml of a
10mg/ml solution) was combined with varying amounts of each
cationic lipid (e.g., 0.4mg DDAB) and the solution was

evaporated to dryness overnight in a Speed Vac Concentrator
(Savant). Liposomes were prepared by resuspending the lipids
in 1 ml sterile deionized water and sonicating on ice using
an Ultrasonics Sonicator (microprobe, 50V setting) until the
solution was almost clear. This typically required 5-10
minutes, with pausing every 10-15 seconds to prevent
overheating. Because applicants observed variability in the
transfection frequencies obtained with different batches of
liposomes sonicated to different extents, a procedure was
adopted of sonicating DDAB/PtdEtn (0.4 mg/ml and 1 mg/ml,
respectively) until a 1:10 dilution in distilled water gave
an O.D. of 0.1 at 540 nm. Liposomes were stored at 4°C and
were stable for at least four months.

Example 2: Transfections

        Plasmid DNA was normally purified by isopycnic
banding in CsCl gradients. However, it was found that crude
DNA preparations which had not been separated from RNA
(minipreps) were also very effective. Cells to be
transfected (typically 5 x 10^5 HeLa or BHK cells) were
plated on 3cm dishes. On the following day they were
infected with vaccinia virus encoding the T7 RNA polymerase
(Fuerst et al, Proc. Natl. Acad. Sci. USA, 83, 8122-8126
(1986)) at a multiplicity of 10 and incubated for 30 minutes
at 37°C in 0.5ml DME. Details of individual transfections
are given in the figures. A typical transfection giving

optimal expression in BHK or HeLa cells was performed as
follows. Five µg of plasmid DNA were added to 1 ml of DMEM
in a polystyrene tube followed by addition 30 µl of
liposomes. This solution was mixed gently and then
incubated at room temperature for 10 minutes to allow
binding of DNA to liposomes. The vTF-7 inoculum was
replaced with the liposome-DNA solution and incubated with
the cells for 3 hours at 37°C in a 5% CO_2 incubator. An
additional 1ml of DME with 10% fetal calf serum was then
added without removing the transfection mixture. It was
found that the DNA and liposome amount can be reduced at
least two fold (2.5 µg DNA and 15 µl liposomes) without
reducing the protein expression level significantly.

For transfection using $CaPO_4$-DNA precipitates,
cells in 6cm dishes were infected with VTF-7 as described
above. After removing the inoculum, 4ml of DME with 5%
fetal bovine serum were added followed by 0.75ml of CaPO4-
precipitated DNA containing 15 µg pARG DNA and 15 µg
sonicated calf thymus DNA carrier (added dropwise). The
$CaPO_4$-DNA suspension was prepared essentially as described
in F.L. Graham and A.J. Van der Eb, <u>Virology</u>, <u>52</u>, 456-467
(1973).

<u>Example 3: Fluorescence Microscopy</u>

Indirect immunofluorescence was carried out on
Hela and BHK cells six hours after transfection as described
in J.K. Rose and J.E. Bergmann, <u>Cell</u>, <u>34</u>, 513-524 (1983)

with the following modifications. Fixed HeLa cells were
incubated with rabbit anti-VSV serum (1:200), followed by
FITC conjugated goat anti-rabbit immunoglobulin G (IgG)
(1:50 dilution; Zymed Laboratories, San Francisco,
California) for detection of cell-surface G protein. For
detection of G protein on the surface of BHK cells, a mouse
monoclonal antibody (I1, L. Lefrancois and D.S. Lyles,
Virology, 121, 157-167 (1982)) was used with an FITC-
conjugated goat anti-mouse second antibody. For
determination of the percent of cells expressing, at least
five random fields of cells were counted (250-500 cells)
using a Nikon Microphot-FX fluorescence microscope equipped
with a planapochromat 40x objective.

Example 4: Flow Cytometry

BHK or HeLa cells were infected with vTF7-3 and
transfected as described above with various ratios of DNA
and cationic liposome suspensions. The cells were removed
from the dish and fixed in suspension at 6 hours post-
transfection. The cells were processed for flow cytometric
analysis as described in M. Whitt, L. Chong and J.K. Rose,
J. Virol., 63, 3569-3578 (1989), except that in some
experiments 0.5% bovine serum albumin was included during
the antibody incubations.

Example 5: Radiolabeling and Immunoprecipitation

Four hours post-transfection, cells were
metabolically labeled by incubation in methionine free DMEM
containing [^{35}S]-methionine (25μCi/0.5 ml) for 30 minutes.
Cells were lysed, and the VSV G protein was
immunoprecipitated with a rabbit anti-VSV antibody as
described in J. K. Rose and J.E. Bergmann, Cell, 34, 513-524
(1983). Immunoprecipitated proteins were analyzed by
electrophoresis in 10% polyacrylamide gels in the presence
of sodium dodecyl sulfate. Dried gels were subjected to
autoradiography. For quantitation of radioactivity in the
bands, films were scanned with a Hoeffer model GS300
densitometer and the peaks were integrated.

Example 6: Use Of Various Cationic Lipids To Form Liposomes
To Mediate DNA Transfection

        To determine if cationic lipids other than DOTMA
could form liposomes that would mediate DNA transfection
into the cytoplasm of animal cells, an experiment was
performed using four commercially available cationic lipids
(see Table 1). These lipids were chosen because they had
long alkyl chains, were relatively insoluble in water, and
thus were not expected to act as strong detergents.
Liposomes were formed by sonication with a constant amount
of the neutral lipid, dioleylphosphatidylethanolamine
(PtdEtn), and the amount of each cationic lipid was varied
as indicated. Th neutral lipid was added because of the

previous report that it enhanced transfection of liposomes

containing DOTMA (Felgner et al, _Proc. Natl. Acad. Sci USA_,

84, 7413-7417 (1987)). The vesicular stomatitis virus (VSV)

glycoprotein (G) has been expressed from cloned DNA

previously and is transported to the cell surface (Rose et

al, _Cell_, **34**, 513-524 (1983)). Plasmid DNA designated pARG

(M. Whitt, L. Chong and J.K. Rose, _J. Virol._, **63**, 3569-3578

(1989)) containing a cDNA clone encoding the VSV G protein

under control of the bacteriophage T7 promoter was mixed

with the preformed liposomes and then added to cells. These

cells had been infected with vTF-7 for 30 minutes prior to

transfection. vTF-7 is a vaccinia virus recombinant which

expresses the bacteriophage T7 RNA polymerase in the

cytoplasm of infected cells (Fuerst et al, _Proc. Natl. Acad.

Sci. USA_, **83**, 8122-8126 (1986)). Six hours after

transfection, cells were fixed with paraformaldehyde,

labeled with fluorescent antibodies recognizing the VSV G

protein, and counted using a fluorescence microscope to

determine the percent of cells expressing the protein.

Results of these initial experiments (Table 1) showed that

all four of the compounds tested were effective in HeLa

cells when used at an appropriate concentration. Two of

these compounds (cetyldimethlyethylammonium bromide and

methylbenzethonium chloride) also caused cell lysis at

higher concentrations. However, only two of the compounds,

namely, stearylamine and dimethyldioctadecylammonium bromide

(DDAB), gave detectable transfection of BHK cells, and DDAB

was far more effective than stearylamine. Because these and

other experiments suggested that DDAB was more effective

than the other compounds on a variety of cells, a more

detailed analysis for only DDAB was conducted.

Table 1. Effectiveness of several cationic lipids in

transfection of HeLa and BHK cells.

		Percent Cells Expressing	
Cationic lipid	(μg/ml)	HeLa	BHK
Cetyldimethylethylammonium bromide	3	70	0
	6	76	0
	12	0*	0*
Dimethyldioctadecyl-ammonium bromide	6	50	30
	12	67	64
Methylbenzethonium chloride	3	23	0
	6	63	0
	12	0*	0*
Stearylamine	0.3	10	0
	3	60	5
	30	1	0.5

Cells were transfected with 5μg pARG DNA bound to liposomes
containing the indicated amounts of cationic lipids and 30
μg PtdEtn as described herein. Percentage of cells
expressing was determined by indirect immunofluorescence
microscopy at 6 hours after transfection. Liposomes formed
with 30 μg/ml dipalmitoylphosphatidylethanolamine and 12
μg/ml of DDAB did not result in transfection of HeLa or BHK
cells.

* indicates cell lysis

        To determine the transfection efficiency of

liposomes containing DDAB on other cell types, several other

cell lines were examined. Transfections into vTF-7 infected

cells were conducted under conditions that were known to be
optimal for BHK and HeLa cells. Determination of the percent
of cells expressing was by indirect immunofluorescence
microscopy. For AtT-20 cells (murine anterior pituitary) and
RK-13 cells (rabbit kidney fibroblast), 50-60 percent of the
cells expressing was obtained. For MDCK cells (Madin-Darby
canine kidney epithelial), about 10% of the cells expressing
were obtained. For other cells such as mouse L-cells and
NIH 3T3 cells, the frequency of cells expressing was usually
only about 5 percent. Variability in results obtained with
cells that were poorly transfected suggested that a
systematic analysis of DNA and lipid concentrations might
well improve the transfection frequencies in these lines.

        In addition to examining the percentage of cells
expressing by direct counting under a fluorescence
microscope, flow cytometry was also used to assess the
accuracy of the direct cell counts and to examine the
distribution of fluorescence intensities for the expressing
cells. Fig. 1 shows examples of flow cytometry profiles for
control (mock-transfected with DDAB liposomes) (Fig. 1A) and
pARG transfected HeLa cells (Fig. 1B). Duplicate 6 cm
dishes containing 2×10^{5} HeLa cells were infected with vTF-
7 and then either transfected with 15μg DNA encoding the VSV
G protein (pARG) and 45 μl liposomes containing 18 μg DDAB
or mock transfected with liposomes only. After six hours,
cells were then fixed, immunostained for cell surface G

protein and analyzed by flow cytometry as described in Whitt et al, _J. Virol._, _63_, 3569-3578, (1989).

The control cells fall in a sharp peak representing background fluorescence, while the majority of transfected cells (85%) are clearly positive for cell-surface expression of the VSV G protein. Using $CaPO_4$-DNA transfection, only 10% of the cells expressing (data not shown) was obtained. The heterogeneity in fluorescence of the positive cells probably reflects delivery of variable amounts of plasmid DNA into the cytoplasm of individual cells. The values obtained by direct counting paralleled those obtained by flow cytometry, but the flow cytometry values were generally somewhat higher. This difference probably reflects the greater sensitivity of the flow cytometry measurements.

Example 7:   Effect of DDAB concentration

Liposomes containing a constant amount of PtdEtn and varying amounts of DDAB were prepared as described herein. Dishes containing 2×10^5 HeLa or BHK cells on coverslips were infected with vTF-7 and then transfected with liposomes containing the indicated amounts of DDAB and 5 μg DNA. The percentage of cells expressing was determined by immunofluorescence microscopy. The results are depicted in Fig. 2. The concentration of PtdEtn was held constant at 30 μg/ml. Liposomes formed with PtdEtn alone gave no

transfection. A final concentration of 12 μg/ml DDAB (30 μl
of liposomes containing 0.4mg/ml DDAB and lmg/ml PtdEtn
added to lml DMEM) appeared optimal and the percentage of
cells expressing dropped off markedly at higher DDAB
concentrations. HeLa cells with liposomes containing only
DDAB were examined and it was found that these were only
about half as effective as those containing PtdEtn (data not
shown).

Example 8: Effect of DNA concentration

The effect of DNA concentration on the
transfection frequency in BHK cells at the optimal lipid
concentration as determined in Fig. 2 was examined. Dishes
containing 2 x 10^5 BHK cells on coverslips were transfected
with the indicated amounts of DNA and 30 μl DDAB liposomes.
The percentage of cells expressing was determined by
immunofluorescence microscopy. The results are depicted in
Fig. 3.

With as little as 0.1 μg of DNA, 18% of the cells
expressing were obtained. This increased to a maximum of
62% with 5 μg of DNA in this experiment and was often as
high as 80-90%. At higher DNA concentrations, there was a
small decrease in number of cells expressing. Although the
decrease appears insignificant in this experiment, more
marked decreases at the high DNA concentrations with some
liposome preparations were observed.

To test the effect of DNA concentration on the
rate of protein synthesis directed by the transfected DNA,
vTF-7 infected cells were transfected with the amounts of
pARG DNA indicated (Fig. 3) and then pulse-labeled cells
with [^{35}S]-methionine at four hours after transfection.
This time was chosen because earlier experiments had shown
that the rate of protein synthesis was already maximal by 4
hours. The VSV G protein was immunoprecipitated from the
lysates and electrophoresed on an SDS-polyacrylamide gel.
To ascertain the effect of DNA concentration on the rate of
VSV G protein synthesis, six dishes containing 5 x 10^5 Hela
cells each were infected with vTF-7 and then transfected
with 30 μl DDAB-liposomes and with certain amounts of DNA.
The rates of VSV G protein synthesis were assessed by pulse-
labeling with [^{35}S]-methionine, immunoprecipitation of the G
protein, and autoradiography after gel electrophoresis.
The results are depicted in Fig. 4.

Quantitation of the radiolabeled protein (Fig. 4)
showed that the rate of protein synthesis reached a maximum
with 5 μg of DNA and dropped off above this level. These
results thus correlate well with those in Fig. 3, showing
that transfection of 5 μg of DNA with 30 μl of DDAB
liposomes gave an optimal percentage of cells expressing.

Example 9: Comparison with DOTMA and CaPO$_4$

To determine how effective liposomes composed of
DDAB were compared to those containing DOTMA, transfections

of vTF-7-infected BHK cells with pARG and quantitated cell surface VSV G protein by flow cytometry were performed. A transfection with $CaPO_4$ precipitated DNA was also included for comparison. Four 6cm dishes each containing 2×10^6 BHK cells were infected with vTF-7 and were mock transfected (Fig. 5A), transfected with 15 μg of pARG DNA and 45 μl of DDAB-liposomes (Fig. 5B), 15μg pARG DNA and 30 μl "LIPOFECTIN"[TM] reagent (DOTMA liposomes) (Fig. 5C), and with 15 μg pARG DNA as a $CaPO_4$ precipitate (Fig. 5D). At six hours after transfection, cells were fixed and stained for cell-surface G protein.

The flow cytometry profiles are shown in Fig. 5. Comparison with Fig. 5A (negative control of mock-transfected cells, DDAB liposomes) showed that DDAB-liposomes (Fig. 5B) gave 95% and DOTMA-liposomes (Fig. 5C) gave 85% of the cells expressing. A $CaPO_4$-precipitate of the same amount of DNA (Fig. 5D) yielded only 30% of the cells expressing. In other experiments DDAB-liposomes gave expression frequencies that were as much as two-fold higher than those obtained with DOTMA-liposomes.

<u>Results</u>

Although liposomes containing all four lipids tested herein were effective in HeLa cells at some concentration, only one, dimethyldioctadecylammonium

bromide, also mediated efficient DNA transfection into the
cytoplasm of a variety of other cells including BHK, RK13,
AtT20 (a pituitary cell line), and MDCK (Madin-Darby canine
kidney), an epithelial cell line. The very high frequency
of transient expression that observed herein (up to 95% of
the cells expressing) is presumably due to the requirement
that the DNA only reach the cytoplasm. In other highly
efficient transient systems such as monkey COS cells (Y.
Gluzman, Cell, 23, 175-182, (1981)) the DNA must also cross
the nuclear membrane to be expressed.

The mechanism by which liposomes containing
positively charged lipids mediate transfection of DNA into
animal cells is not presently understood. The DNA
undoubtedly binds to the positively charged surface of the
liposome, and residual positive charge then presumably
mediates binding to negatively charged sialic acid residues
on cell surfaces. The decrease that was observed in
transfection frequencies at high DNA concentrations (Fig. 4)
might be attributed to saturation of the positive charge on
the liposomes.

Felgner et al, Proc. Natl. Acad. Sci. USA, 84,
7413-7417 (1987) presented evidence suggesting that
liposomes containing DOTMA fuse with the plasma membrane.
Because the plasmid DNA should be located on the outside of
the liposomes, one would anticipate that fusion of the
liposome with the plasma membrane would leave the DNA on the

cell surface. An alternativ possibility is that DNA bound
to liposomes is taken up by endocytosis and that some
fraction of the DNA is then released into the cytoplasm by
an unknown mechanism. Given the uncertainty of how the DNA
enters the cytoplasm, it is premature to speculate on why
three of the cationic lipids tested worked well on HeLa
cells, but not on BHK or other cells that were tested. One
or more of these lipids may well be more effective than DDAB
on some cell types.

The level of production of VSV G protein achieved
in BHK cells was quantitated using DDAB transfection and the
vaccinia/T7 system. Using immunoblots standardized with
known amounts of VSV proteins, it was found that BHK cells
transfected with pARG synthesized the equivalent of 3×10^6
molecules VSV G protein per cell within six hours after
transfection. The level of translation of mRNA transcribed
by the T7 polymerase in vaccinia infected cells appears
limited by the efficiency with which vaccinia virus enzymes
cap the transcripts. The newly described use of the cap-
independent translation initiation signal from EMC virus in
conjunction with the vaccinia/T7 system will undoubtedly
allow even higher levels of expression (O. Elroy-Stein, T.
R. Fuerst and B. Moss, Proc. Natl. Acad. Sci. USA, 86, 6126-
6130, (1989)).

An important aspect of the vaccinia/T7 system
combined with DDAB mediated transfecti n, is that it permits

a high efficiency of simultaneous expression of two or more
genes. Using indirect immunofluorescence to monitor
expression of two DNAs transfected simultaneously into HeLa
cells, it was found that all (>95%) of the expressing cells
were expressing both proteins at very similar levels. This
feature of the system is very important to quantitative
studies on protein-protein interactions where similar levels
of each protein need to be made in all cells.

Given the high efficiency of DDAB containing
liposomes in mediating cytoplasmic transfection in the
vaccinia/T7 system, it is surprising that liposomes
containing DDAB were reported to be ineffective in mediating
transfection into cell nuclei (Felgner et al, _Proc. Natl._
Acad. Sci. USA, _84_, 7413-7417 (1987) and F.L. Felgner and
G.M. Reingold, _Nature_, _337_, 387-389 (1989)). It is
conceivable that liposomes containing DDAB are efficient
only at delivering DNA into the cytoplasm. Arguing against
this possibility is the fact that transient nuclear
expression using DDAB containing liposomes and an SV40-based
vector in monkey COS cells was observed. Transient nuclear
expression of DNA in BHK cells was also observed.
Transfection of DNA with liposomes containing DDAB is
clearly ideal for many applications involving the
vaccinia/T7 hybrid system, and will prove useful in many
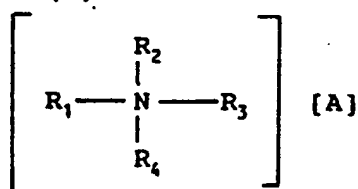other applications as well.

It will be appreciated that the instant specification is set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

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WHAT IS CLAIMED IS:

1.   A reagent for introducing a nucleic acid into an animal cell comprising

a.   a neutral lipid and

b.   a cationic lipid selected from the group consisting of

(1)   an ammonium salt of the formula

$$\left[\begin{array}{c} R_2 \\ | \\ R_1 - N - R_3 \\ | \\ R_4 \end{array}\right] \quad [A]$$

, wherein R_1 is a straight hydrocarbon chain of C_{14} to C_{18} that is saturated or unsaturated,

R_2, R_3 and R_4 are, independently of each other, hydrogen, a straight hydrocarbon chain of C_1 to C_{18} that is saturated or unsaturated or an aryl, and A is an anion,

(2)   an amine of the formula

$$R_5 - N - R_7,$$
with R_6 above N
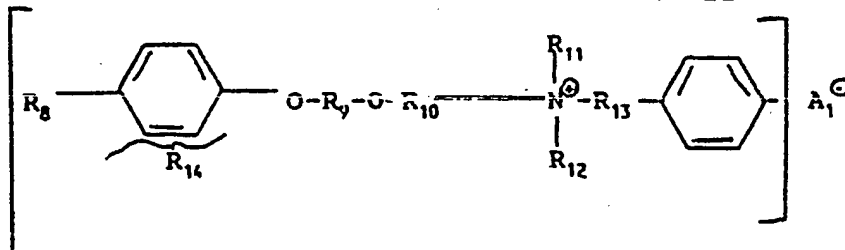
wherein R_5 is a straight chain of C_{14} to C_{18} that is saturated or unsaturated and

R_6 and R_7 are, independently of each other, hydrogen or C_1 to C_5 alkyl, and

(3)   a benzethonium salt of the formula

$$\left[R_8 - \text{(ring)} - O - R_9 - O - R_{10} - N^{\oplus} - R_{13} - \text{(ring)} \right] A_1^{\ominus}$$
with R_{14}, R_{11}, R_{12} substituents

, wherein R_8 is a straight chain or branched C_1-C_{10}-alkyl,

R_9 is a C_1-C_{10}-alkyl,

R_{10} is a C_1-C_{10}-alkyl,

R_{11} is a C_1-C_{10}-alkyl,

R_{12} is a C_1-C_{10}-alkyl,

R_{13} is a C_1-C_{10}-alkyl,

R_{14} is a C_1-C_{10}-alkyl and $A_1^{\ominus}$ is an anion.

2. A reagent according to claim 1, wherein the neutral lipid is dioleoylphosphatidylethanolamine.

3. A reagent according to claim 1, wherein the neutral lipid is phospholipid-related materials such as lecithin, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphinogomyelin, cephalin, cardiolipin, phosphatidic acid, cerebrosides, acetylphosphate, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dioleoylphosphatidyl-glycerol, dipalmitoylphosphatidylglycerol, dioleoyl-phosphatidylethanolamine, palmitoyloleoylphosphatidyl-choline, palmitoyloleoylphosphatidylethanolamine, dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, diheptadecanoylphosphatidyl-ethanolamine, dilauroylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, distearoylphosphatidyl-ethanolamine, beta-linoleoyl-gamma-palmitoylphosphatidyl-

ethanolamine or beta-oleoyl-gamma-palmitoylphosphatidyl-
ethanolamine.

4.   A reagent according to claim 1, wherein the
ammonium salt is dimethyldioctadecylammonium bromide.

5.   A reagent according to claim 1, wherein the
ammonium salt is cetyldimethylethylammonium bromide.

6.   A reagent according to claim 1, wherein the
amine is stearylamine.

7.   A reagent according to claim 1, wherein the
benzethonium salt is methylbenzethonium chloride.

8.   A process for introducing a nucleic acid into
an animal cell comprising (a) contacting a nucleic acid with
a reagent according to claim 1 to form a liposome or lipid
micelles and (b) contacting the resultant liposome or lipid
micelles from step (a) with an animal cell.

9.   A complex between a nucleic acid and the
reagent of claim 1.

10.   The reagent of claim 9, further comprising a
liposome.

11.   The reagent of claim 9, further comprising
lipid micelles.

AMENDED CLAIMS

, wherein R_8 is a straight chain or branched C_1-C_{10}-alkyl,

R_9 is a C_1-C_{10}-alkyl,

R_{10} is a C_1-C_{10}-alkyl,

R_{11} is a C_1-C_{10}-alkyl,

R_{12} is a C_1-C_{10}-alkyl,

R_{13} is a C_1-C_{10}-alkyl,

R_{14} is a C_1-C_{10}-alkyl and $A_1^{\ominus}$ is an anion.

2.   A reagent according to claim 1, wherein the
neutral lipid is dioleoylphosphatidylethanolamine.

3.   A Reagent according to Claim 1,
wherein the neutral lipid is a phospholipid-related
material selected from the group consisting of
lecithin, lysolecithin, lysophosphatidylethanolamine,
phosphatidylserine, phosphatidylinositol, sphinogomyelin,
cephalin, cardiolipin, phosphatidic acid, cerebrosides,
acetylphosphate, dioleoylphosphatidylcholine,
dipalmitoylphosphatidylcholine, dioleoylphosphatidyl-
glycerol, dipalmitoylphosphatidylglycerol, dioleoyl-
phosphatidylethanolamine, palmitoyloleoylphosphatidyl-
choline, palmitoyloleoylphosphatidylethanolamine,
dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)
cyclohexane-1-carboxylate, diheptadecanoylphosphatidyl-
ethanolamine, dilauroylphosphatidylethanolamine,
dimyristoylphosphatidylethanolamine, distearoylphosphatidyl-
ethanolamine, beta-linoleoyl-gamma-palmitoylphosphatidyl-

ethanolamine and beta-oleoyl-gamma-palmitoylphosphatidyl-
ethanolamine.

4. A reagent according to claim 1, wherein the
ammonium salt is dimethyldioctadecylammonium bromide.

5. A reagent according to claim 1, wherein the
ammonium salt is cetyldimethylethylammonium bromide.

6. A reagent according to claim 1, wherein the
amine is stearylamine.

7. A reagent according to claim 1, wherein the
benzethonium salt is methylbenzethonium chloride.

8. A process for introducing a nucleic acid into
an animal cell comprising (a) contacting a nucleic acid with
a reagent according to claim 1 to form a liposome or lipid
micelles and (b) contacting the resultant liposome or lipid
micelles from step (a) with an animal cell.

9. A complex between a nucleic acid and the
reagent of claim 1.

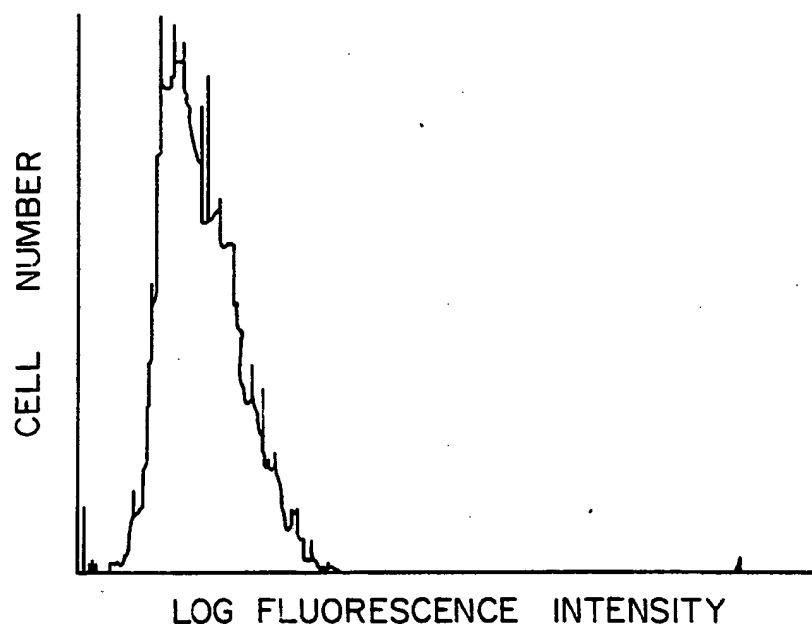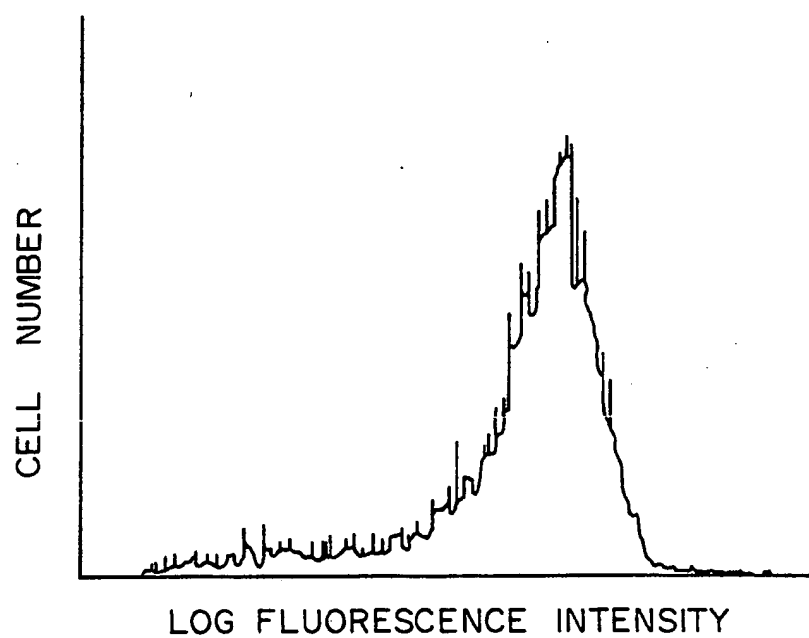10. The reagent of claim 9, further comprising a
liposome.

FIG. 1A

FIG. 1B

FIG. 2

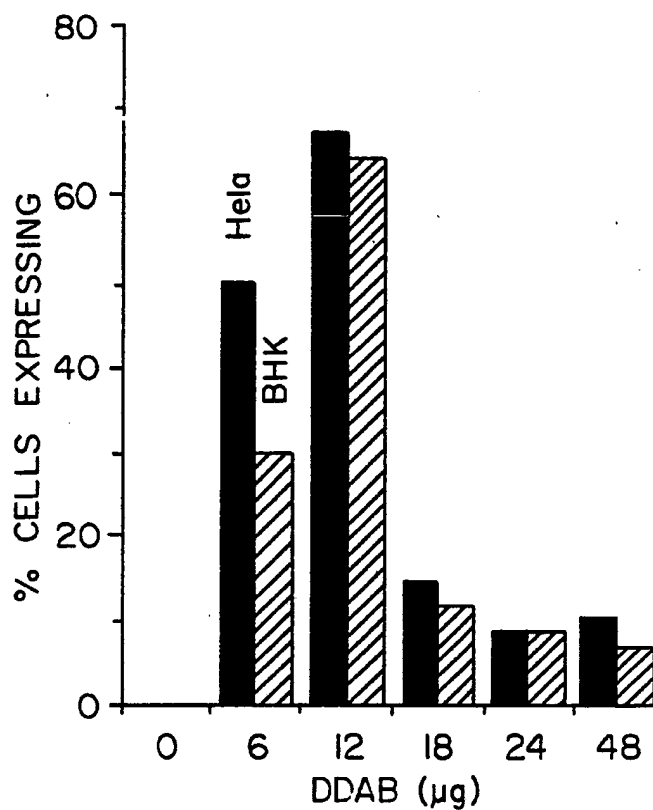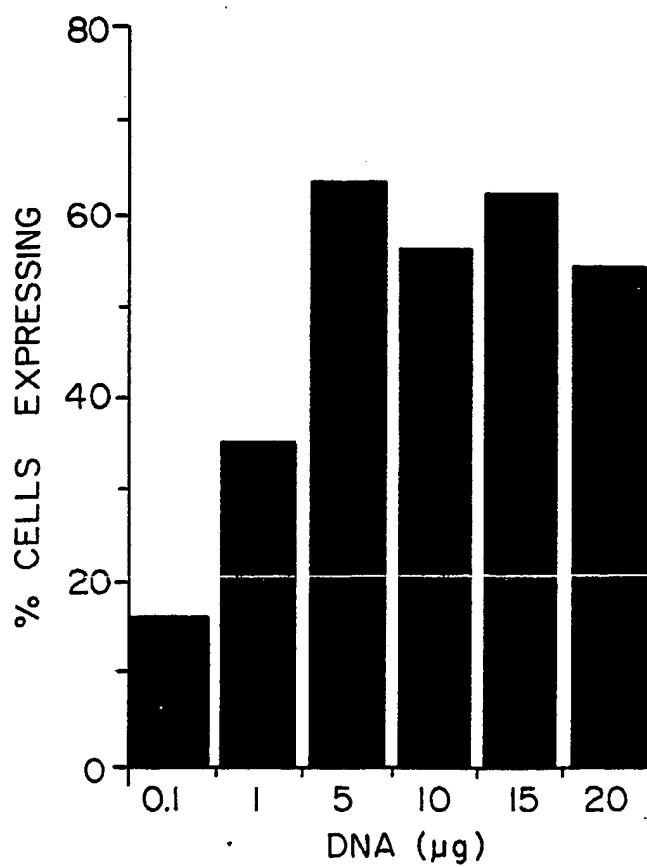FIG. 3

FIG. 4

FIG. 5A

CONTROL

CELL NUMBER

LOG FLUORESCENCE INTENSITY

FIG. 5B

DDAB

CELL NUMBER

LOG FLUORESCENCE INTENSITY

FIG. 5C

FIG. 5D

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

I PC. CL.(5): C07H 21/02; C12N 5/00; A61K 37/22; C12Q 1/68
U.S. Cl.: 536/27; 435/6; 436/71; 435/240.2; 424/450

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S. Cl.:	536/27; 435/6; 436/71; 435/240.2; 424/450

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
A	Proceedings of the National Academy of Sciences, Volume 86, issued September 1989, Behr et al., "Efficient gene transfer into Mammalian primary Endocrine Cells with lipopoly-amine-coated DNA," pages 6982-6986, see entire document.	1-11
A	Nature, Volume 337, issued 26 January 1989, Felgner et al., "Cationic liposome- Mediated Transfection," pages 387-388, see entire document.	1-11
A	Proceedings of the National Academy of Sciences, Volume 84, issued November 1987, Felgner et al., "Lipofection: A Highly Efficient, Lipid-Mediated DNA- Transfection," pages 7413-7417, see entire documents.	1-11

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

02 MAY 1991

Date of Mailing of the International Search Report

20 JUN 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

GARY L. KUNZ

Form PCT SA 210 (second sheet) (rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y,E	US,A, 4,946,787, (EPPSTEIN ET AL.) 07 AUGUST 1990, SEE ENTIRE DOCUMENT.	1-11
Y	US,A, 4,897,355, (EPPSTEIN ET AL.) 30 JANUARY 1990, SEE ENTIRE DOCUMENT.	1-11